Nutritional and hormonal regulation of malic enzyme synthesis in rat mammary gland

María F. LOBATO, Manuel ROS, Francisco J. MORENO and Josefa P. GARCÍA-RUÍZ

Departamento de Bioquímica y Biología Molecular, Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma de Madrid, 28049 Madrid, Spain

Cytosolic malic enzyme was purified from rat mammary gland by L-malate affinity chromatography. The pure enzyme obtained was used to produce a specific antiserum in a rabbit. Relative synthesis of malic enzyme in the mammary gland of mid-lactating rats was 0.097%, measured by labelling the enzyme in isolated acini. When food was removed, malic enzyme synthesis decreased to 35% and 20% of the control value at 4 and 6 h respectively. Incorporation of [³H]leucine into soluble proteins was constant during the first 6 h of starvation. When lactating rats (maintained with their pups) were starved for 24 h and then re-fed, the relative rate of enzyme synthesis increased 2.5-, 4-, and 4.5-fold at 3 h, 6 h and 18 h respectively after initiation of re-feeding. The relative rate of malic enzyme synthesis was about 50% of normal at 15 h after weaning, whereas the rate of synthesis of soluble proteins did not change. Administration of bromocryptine or adrenalectomy of lactating rats decreased the relative rate of synthesis of malic enzyme by 40% or 30% respectively; these effects were counteracted by hormone supplementation. Hormone therapy also caused an increase in the rate of incorporation of [³H]leucine into soluble proteins and in malic enzyme activity.

INTRODUCTION

Cytoplasmic malic enzyme (EC 1.1.1.40), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) provide reducing equivalents in the form of NADPH for the synthesis *de novo* of fatty acids in lipogenic tissues. Hormones, nutritional factors and states of cellular differentiation regulate the concentration of malic enzyme in mammalian and avian liver (Goodridge & Adelman, 1976; Towle *et al.*, 1980; Siddiqui *et al.*, 1981; Miksicek & Towle, 1982; Goodridge *et al.*, 1984). At the peak of lactation, the mammary gland is the most active site of lipogenesis in the rat (Frenkel, 1975; Volpe & Vagelos, 1976; Williamson, 1980; Cubero *et al.*, 1983; Careche *et al.*, 1985). On the basis of measurements in isolated acini, the rate of lipid synthesis is dependent on the hormonal and nutritional state of the animal (Williamson, 1980; Cubero *et al.*, 1983; Careche *et al.*, 1985). Variations in the rate of lipogenesis are accompanied by parallel changes in the specific activities of the enzymes involved in NADPH production. We report here a new and simple method for the purification of malic enzyme and the conditions of how to use isolated acini to label the protein that is being synthesized in the mammary gland. Antiserum raised against pure malic enzyme was used to measure the relative rate of malic enzyme synthesis under different nutritional and hormonal conditions.

MATERIALS AND METHODS

Materials

Collagenase was type CLS III from *Clostridium histolyticum* (Worthington); bovine serum albumin (fraction V) (Armour Pharmaceutical Co.), AH (1,6-diaminohexane)-Sepharose 4B (Pharmacia), L-[³H]leucine (Amersham International) and electrophoresis materials (Bio-Rad) were purchased from the indicated suppliers. All other reagents were obtained from Sigma, Boehringer

Mannheim or Merck. Bromocryptine mesylate was generously given by Dr. E. Fluckiger, Sandoz, Basle, Switzerland.

Animals

Wistar rats (14–17 days *post partum*, peak lactation) kept with eight to ten pups and weighing 280–300 g were used in all experiments. Rats starved for 24 h (09:00–09:00 h; the light period was 07:00–19:00 h) were re-fed with a single intragastric dose of 6 ml of 0.25 M-sucrose, followed by chow pellets *ad libitum*. Premature weaning was accomplished by removing newborns from rats at mid-lactation. Adrenalectomy was performed under diethyl ether anaesthesia in lactating rats 2 days after parturition; the animals received 0.9% NaCl (saline) in their drinking water. Dexamethasone was subcutaneously administered 4 days after surgery at 0.8 mg/day for 2 days. Control rats received saline for 2 days. Prolactin deficiency was induced in lactating rats by subcutaneous injection of 2-bromo- α -ergocryptine mesylate (10 mg/kg body wt.) (Baxter & Coore, 1978). Some bromocryptine-treated animals were subcutaneously injected with sheep prolactin (15 mg/kg body wt.) (McNeillie & Zammit, 1982). Bromocryptine and prolactin were administered once per day for 2 days.

Purification of malic enzyme and antibody production

Inguinal mammary glands (6–8 g) were excised from rats at mid-lactation and were homogenized in 2 vol. of 0.25 M-sucrose/50 mM-Tris/HCl/50 mM-KCl/1 mM-EDTA/1 mM-dithiothreitol, pH 7.4, in a Brinkman Polytron for 3 $\times$ 15 s at setting 8. Supernatants were obtained by centrifugation at 105000 g for 60 min and had a specific activity of about 0.23 unit/mg of protein. They were passed over a Sephadex G-100 column (2.5 cm $\times$ 70 cm) equilibrated with 10 mM-Tris/HCl/2 mM-EDTA, pH 7.6. Malic enzyme was eluted in the second part of the first major protein peak. The next step

Fig. 1. SDS/polyacrylamide-gel electrophoresis of malic enzyme purified from mammary gland of lactating rats

The running gel contained 9% polyacrylamide (Maizel, 1971); 5–10 μ g samples were applied. Lanes: (1) 105000 g supernatant from mammary gland of lactating rats, 15 days after parturition; (2) Sephadex G-100 eluate; (3) eluate after malate affinity chromatography; (4) malic enzyme after gel electroelution; (5) and (6) were protein markers, bovine serum albumin (M_r 66000) and pyruvate kinase (M_r 60000) respectively.

was affinity chromatography on L-malate-AH-Sepharose 4B. The affinity gel was synthesized by incubating 4 g of AH-Sepharose 4B (properly swelled and washed) with 100 mg of L-malate and 100 mg of carbodi-imide at pH 6.5 for 24 h at room temperature. After coupling, the gel was extensively washed before use and equilibrated with the same buffer as used in the above Sephadex filtration step. Malic enzyme activity was eluted with 30 mM-L-malate, pH 7. About 50% of the malic enzyme was retained on the column and eluted with malate. The remaining contaminating proteins were eliminated by electrophoretic elution of the band from a preparative-7%-polyacrylamide gel after electrophoresis under non-denaturing conditions (Maizel, 1971). Malic enzyme purified by this procedure gave a single band on SDS/polyacrylamide-gel electrophoresis (Fig. 1). The purified enzyme was used to immunize a rabbit (Clausen, 1971). γ -Globulin was prepared by two successive 40%-satd.-(NH₄)₂SO₄ fractionations of the rabbit antiserum. The final pellet was dissolved in half of the volume

of the serum from which it was derived and dialysed against large volume of 10 mM-sodium phosphate/15 mM-NaCl, pH 7.4. The antibody preparation was assayed by double immunodiffusion, giving a single precipitin line (Ouchterlony, 1953). Malic enzyme activity was determined by the method of Hsu & Lardy (1966). Protein was assayed by the method of Lowry *et al.* (1951).

Preparation of acini

Inguinal mammary glands (5–8 g) were removed, chopped and washed as described by Katz *et al.* (1974) and Robinson & Williamson (1977), and digested in 35 ml of Krebs & Henseleit (1932) saline containing collagenase (1 mg/ml) and 10 mM-glucose. Oxygen was supplied by bubbling O₂/CO₂ (19:1) during the 1 h digestion. The acini (0.5 ml) were incubated in a silicone-treated 50 ml Erlenmeyer flask in a total volume of 2.0 ml containing 1 mM of each of the 19 protein amino acids and 50 μ Ci of L-[³H]leucine/ml for 30 min. Each experiment was carried out in duplicate at 37 °C under O₂/CO₂ (19:1) in a metabolic incubator with shaking at 200 strokes/min. The reactions were stopped by placing the flasks on ice.

Relative rate of enzyme synthesis

After the cells were incubated with L-[³H]leucine, the tissue was collected by centrifugation at 800 g for 10 min at room temperature and then washed with 2 $\times$ 2 ml of Krebs-Henseleit saline containing 5 mM-leucine. The final pellet was suspended in 1 ml of 30 mM-Tris/HCl/0.25 M-sucrose/1 mM-EDTA/1 mM-dithiothreitol/5 mM-L-leucine, pH 7.6, and homogenized for 20 s in a Brinkman Polytron at setting 8. Enzyme synthesis was measured by direct immunoprecipitation of malic enzyme from a 105000 g supernatant (Hopgood *et al.*, 1973). Immunoprecipitations were carried out with 90–180 munits of enzyme activity and sufficient antiserum to precipitate 120% of the total malic enzyme activity present. When malic enzyme activity in the supernatant was less than 90 munits, crude liver extracts containing malic enzyme activity were added as carriers. The immunoprecipitation reactions were incubated for 60 min at 37 °C, then for 18 h at 4 °C, and the immunoprecipitate obtained was collected and washed as described by Miksicek & Towle (1982). No malic enzyme activity was detected in the supernatant after its immunoprecipitation. The immunoprecipitate was dissociated and subjected to electrophoresis in SDS/9%-polyacrylamide gels (Maizel, 1971). The malic enzyme band was cut out of the gel and incubated with 0.2 ml of H₂O₂ (30%) at 74 °C for 24 h. Radioactivity incorporated into soluble protein was determined in 25 μ l of a 105000 g supernatant precipitated with trichloroacetic acid. Results are expressed as c.p.m./mg of protein.

RESULTS AND DISCUSSION

We decided to analyse the effects of starvation, weaning and lactogenic hormones on malic enzyme synthesis, because weaning or starvation of lactating rats decreased malic enzyme activity and the activity of the NADPH-producing enzymes of the hexose monophosphate shunt in mammary gland (Korsrud & Baldwin, 1972; Mayer, 1978; Cubero *et al.*, 1983; Careche *et al.*, 1985). Isolated mammary-gland acini were used to label proteins because they are convenient and yield more

Fig. 2. Time course of the relative rates of malic enzyme synthesis in rat mammary gland caused by starvation and re-feeding

Rats were killed at the times specified. Acini were isolated and labelled as described in the Materials and methods section. The values are means $\pm$ S.E.M. for four or more determinations.

reproducible and more accurate results than with slices of mammary gland. When acini were pulse-labelled with [3 H]leucine, incorporation of label into soluble protein was 5 times higher than in slices. Incorporation of [3 H]leucine into soluble protein was linear with respect to time (0–30 min) under the conditions that we used (results not shown).

The effect of starvation and re-feeding on the relative rate of malic enzyme synthesis (ratio c.p.m./mg of protein in malic enzyme to c.p.m./mg of protein in soluble protein) is shown in Fig. 2. At 4 h after removal of food, the relative rate of enzyme synthesis had decreased to 35% of control (mid-lactating rats). In contrast, enzyme activity had not changed, nor had incorporation of radiolabel into soluble protein [0 h, $4.08(\pm 0.51) \times 10^6$; 4 h, $4.2(\pm 0.2) \times 10^6$ c.p.m./mg of protein]. By 6 h, relative synthesis of malic enzyme was only 20% of control, enzyme activity had decreased by 20% and incorporation of [3 H]leucine into soluble protein remained unchanged from control values [$3.99(\pm 0.8) \times 10^6$ c.p.m./mg of protein]. After 24 h of starvation, synthesis of malic enzyme was almost undetectable, and enzyme activity and the rate of incorporation of [3 H]leucine into soluble protein had decreased to 50% of the control value [$2.3(\pm 0.6) \times 10^6$ c.p.m./mg of protein]. When lactating rats starved for 24 h, were then (maintained with their pups) re-fed, the relative rate of enzyme synthesis increased 2.5-, 4- and 4.5-fold at 3, 6 and 18 h respectively after initiation of re-feeding. Similar changes occurred in enzyme activity (Careche *et al.*, 1985). The rate of [3 H]leucine incorporation into soluble protein also increased after re-feeding, to $2.3(\pm 0.1)$, $3.4(\pm 0.08)$ and $2.81(\pm 0.3) \times 10^6$ c.p.m./mg of protein at 3, 6 and 18 h respectively. Thus malic enzyme synthesis was restored to about 72% of normal after 18 h of re-feeding, whereas that of soluble proteins returned to about 65% of normal.

Owing to the difficulties encountered in measuring the rate of degradation of malic enzyme in the gland, we do not know its half-life, nor whether or not it is altered in the different situations studied. Taking into account the experiments with the hepatic enzyme, showing that the rate of degradation of the enzyme protein plays a minor role in controlling its concentration (Goodridge & Adelman, 1976), we can make the assumption that mammary gland behaves similarly. Then the results indicate that changes in the activity of malic enzyme correlate with and are probably caused by changes in the relative rate of synthesis of malic enzyme. Serum insulin concentration, markedly altered in lactating rats by starvation/re-feeding (Robinson *et al.*, 1978), may be involved in the variations of the relative rate of enzyme synthesis shown.

The effect of weaning on the relative synthesis of malic enzyme was also assessed. Removal of pups for 6, 15 or 48 h caused the relative rate of malic enzyme synthesis to decrease to 83, 40 or 3% respectively of the rate found in peak-lactating rats (Fig. 3). These results are consistent with the decrease in specific activity of malic enzyme caused by natural or premature weaning (Cubero *et al.*, 1983). The cells maintained a nearly steady [3 H]leucine incorporation into soluble protein during the first 15 h of weaning, but this decreased significantly at 48 h after weaning. Since the alterations observed during starvation/re-feeding could be linked to changes in serum insulin concentration, the decrease on weaning could be the result of a more complex situation. Capillary closure is known to occur in the mammary gland of rats after 36–48 h of milk accumulation. The rate of delivery of hormones to the tissue will be drastically decreased and it is concluded that this, rather than systemic hormone concentrations, is of importance in controlling receptor concentrations in the gland (Moore & Forsyth, 1980). Similar to the

Fig. 3. Time course of the relative rate of malic enzyme synthesis during premature weaning

Acini were isolated and labelled as described in the Materials and methods section. The values are means $\pm$ S.E.M. ($n = 3-5$): ●, malic enzyme; ▲, soluble protein.

starvation/re-feeding effect, the decrease in malic enzyme activity occurred after initiation of weaning, correlating with and possibly caused by changes in the relative rate of synthesis of malic enzyme.

We next assessed the effects of prolactin and glucocorticoids on malic enzyme synthesis in mammary gland (Table 1). Lactating rats were either treated with bromocryptine, to decrease plasma prolactin, or adrenalectomized. Bromocryptine caused the enzyme activity, the soluble protein synthesis and the malic enzyme synthesis to decrease by 43, 73 and 80% respectively. Sheep prolactin partially reversed the effects of bromocryptine. Adrenalectomy of lactating rats caused the malic enzyme activity, the synthesis of soluble protein and the relative synthesis of malic enzyme to decrease by 43, 24 and 41% respectively. Dexamethasone administration to adrenalectomized animals stimulated

the synthesis of protein and malic enzyme 2.6- and 3.4-fold respectively relative to adrenalectomized controls, values which were twice those of untreated normal rats. These results suggest that prolactin and glucocorticoids regulate the amounts of cytosolic malic enzyme, but the effect is not sufficiently marked to account for the large and selective change in malic enzyme synthesis caused by starvation/re-feeding or weaning.

Our results provide evidence that the high malic enzyme activity present in mammary gland during lactation can be due to high extents of enzyme synthesis. In consequence of its role as a NADPH-producing system, the regulation of malic enzyme synthesis is closely coupled to the rate of lipogenesis and could play an important role in the regulation of fatty acid synthesis in the mammary gland. The activity of mammary acetyl-CoA carboxylase changes in similar manner in response to starvation, weaning or bromocryptine treatment (McNeillie & Zammit, 1982). The large and selective change observed in malic enzyme synthesis caused by starvation/re-feeding or weaning and the decreases observed under hypoprolactinaemia or adrenalectomy are now being analysed in an 'in vitro' system.

We thank Dr. J. N. Fain and Dr. Alan G. Goodridge for stimulating discussions during the preparation of the manuscript. We are grateful to Mr. Javier Palacin for his skilful assistance with the animals and Mrs. Carmen Luengo for her technical assistance. This work was supported by a grant from 'Comision Asesora de Investigacion Cientifica y Tecnica' and a grant from 'Fondo de Investigaciones Sanitarias'.

REFERENCES

- Baxter, M. A. & Coore, H. G. (1978) *Biochem. J.* **174**, 553-561
- Careche, M., Lobato, M. F., Ros, M., Moreno, F. J. & Garcia-Ruiz, J. P. (1985) *Horm. Metab. Res.* **17**, 226-229
- Clausen, J. (1971) *Immunochemical Techniques for the Identification and Estimation of Macromolecules*, American Elsevier, New York
- Cubero, A., Ros, M., Lobato, M. F., Garcia-Ruiz, J. P. & Moreno, F. J. (1983) *Enzyme* **30**, 38-47
- Frenkel, R. (1975) *Curr. Top. Cell. Regul.* **9**, 157-181
- Goodridge, A. G. & Adelman, T. G. (1976) *J. Biol. Chem.* **251**, 3027-3032
- Goodridge, A. G., Fish, J. & Glynias, M. J. (1984) *Arch. Biochem. Biophys.* **224**, 54-63

Table 1. Effects of bromocryptine administration and adrenalectomy on the relative rate of synthesis of malic enzyme purified from mammary gland of lactating rats

Each value is the mean $\pm$ S.E.M. for the number of experiments indicated in parentheses. Acini were isolated and labelled for the determination of radioactive leucine incorporation into soluble protein and into malic enzyme as described in the Materials and methods section.

	Activity of malic enzyme (units/mg of protein)		[³ H]Leucine incorporation (c.p.m./mg of protein) into		Relative rate of malic enzyme synthesis (%)
			Soluble protein ($\times 10^{-6}$)	Malic enzyme	
Control	0.21 $\pm$ 0.02 (12)		4.3 $\pm$ 0.35	3576 $\pm$ 400	0.083
Bromocryptine	0.12 $\pm$ 0.006 (3)		1.2 $\pm$ 0.20	718 $\pm$ 100	0.052
Bromocryptine + prolactin	0.18 $\pm$ 0.01 (3)		2.6 $\pm$ 0.32	1802 $\pm$ 216	0.069
Adrenalectomy	0.12 $\pm$ 0.005 (3)		3.3 $\pm$ 0.34	2127 $\pm$ 264	0.064
Adrenalectomy + dexamethasone	0.14 $\pm$ 0.03 (3)		8.9 $\pm$ 1.80	7209 $\pm$ 825	0.081

- Hopgood, M. F., Ballard, F. J., Reshef, L. & Hanson, R. W. (1973) *Biochem. J.* **134**, 445–453
- Hsu, R. J. & Lardy, H. A. (1966) *J. Biol. Chem.* **242**, 520–539
- Katz, J., Wals, P. A. & Van de Velde, R. L. (1974) *J. Biol. Chem.* **249**, 7348–7357
- Korsrud, G. O. & Baldwin, R. L. (1972) *Can. J. Biochem.* **50**, 386–391
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maizel, J. V. Jr. (1971) *Methods Virol.* **5**, 180–244
- Mayer, R. J. (1978) *Vitam. Horm. (N.Y.)* **36**, 101–163
- McNeillie, E. M. & Zammit, V. A. (1982) *Biochem. J.* **204**, 273–280
- Miksicek, R. J. & Towle, H. C. (1982) *J. Biol. Chem.* **257**, 11829–11835
- Moore, B. P. & Forsyth, I. A. (1980) *Nature (London)* **284**, 77–78
- Ouchterlony, O. (1953) *Acta Pathol. Microbiol. Scand.* **32**, 231–235
- Robinson, A. M. & Williamson, D. H. (1977) *Biochem. J.* **164**, 153–159
- Robinson, A. M., Girard, J. R. & Williamson, D. H. (1978) *Biochem. J.* **176**, 343–346
- Siddiqui, V. A., Goldflam, T. & Goodridge, A. G. (1981) *J. Biol. Chem.* **256**, 4544–4548
- Towle, H. C., Mariachu, C. N. & Oppenheimer, J. H. (1980) *Biochemistry* **19**, 579–585
- Volpe, J. J. & Vagelos, P. R. (1976) *Physiol. Rev.* **56**, 339–417
- Williamson, D. H. (1980) *FEBS Lett* **117**, K93–K105

Received 13 November 1985/17 January 1986; accepted 3 February 1986